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I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 0339 for a patent by MEDVET SCIENCE PTY LTD as filed on 13 May 1999.

WITNESS my hand this  
Twenty-sixth day of October 2005

A handwritten signature in dark ink, appearing to read 'J. Peisker'.

**JANENE PEISKER**  
**TEAM LEADER EXAMINATION**  
**SUPPORT AND SALES**



MEDVET SCIENCE PTY. LTD.

**A U S T R A L I A**

**Patents Act 1990**

**PROVISIONAL SPECIFICATION**

for the invention entitled:

"Novel Therapeutic Molecules and Uses Thereof"

The invention is described in the following statement:

- 1A -

## NOVEL THERAPEUTIC MOLECULES AND USES THEREOF

### FIELD OF THE INVENTION

5 The present invention relates generally to novel protein molecules and to derivatives, analogues, chemical equivalents and mimetics thereof capable of modulating cellular activity and, in particular, modulating cellular activity via the modulation of signal transduction. More particularly, the present invention relates to human sphingosine kinase and to derivatives, analogues, chemical equivalents and mimetics thereof. The present invention also contemplates  
10 genetic sequences encoding said protein molecules and derivatives, analogues, chemical equivalents and mimetics thereof. The molecules of the present invention are useful in a range of therapeutic, prophylactic and diagnostic applications.

### BACKGROUND OF THE INVENTION

15

Bibliographic details of the publications referred numerically in this specification are collected at the end of the description.

Sphingosine kinase is a key regulatory enzyme in a variety of cellular responses. Its activity can  
20 affect inflammation, apoptosis and cell proliferation, and thus it is an important target for therapeutic intervention.

Sphingosine-1-phosphate is known to be an important second messenger in signal transduction [1]. It is mitogenic in various cell types [2,3] and appears to trigger a diverse range of important  
25 regulatory pathways including; prevention of ceramide-induced apoptosis [4], mobilisation of intracellular calcium by an  $IP_3$ -independant pathway, stimulation of DNA synthesis, activation of mitogen-activated protein (MAP) kinase pathway, activation of phospholipase D, and regulation of cell motility (for reviews see [1,3,5]).

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Recent studies [6] have shown that sphingosine-1-phosphate is an obligatory signalling intermediate in the inflammatory response of vascular endothelial cells to tumour necrosis factor- $\alpha$  (TNF $\alpha$ ). In spite of its obvious importance, very little is known of the mechanisms that control cellular sphingosine-1-phosphate levels. It is known that sphingosine-1-phosphate levels in the cell are mediated largely by its formation from sphingosine by sphingosine kinase, and to a lesser extent by its degradation by endoplasmic reticulum-associated sphingosine-1-phosphate lyase and sphingosine-1-phosphate phosphatase [3]. Basal levels of sphingosine-1-phosphate in the cell are generally low, but can increase rapidly and transiently when cells are exposed to mitogenic agents. This response appears correlated with an increase in sphingosine kinase activity in the cytosol and can be prevented by addition of the sphingosine kinase inhibitory molecules *N,N*-dimethylsphingosine and DL-*threo*-dihydrosphingosine. This indicates that sphingosine kinase is an important molecule responsible for regulating cellular sphingosine-1-phosphate levels. This places sphingosine kinase in a central and obligatory role in mediating the effects attributed to sphingosine-1-phosphate in the cell.

Accordingly, there is a need to identify and clone novel sphingosine kinase molecules to facilitate the progression towards the more sensitive control of intracellular signal transduction via, for example, the elucidation of the mechanism controlling the expression and enzymatic activity of sphingosine kinase thereby providing a platform for the development of interventional therapies to regulate the expression or activity of sphingosine kinase. In work leading up to the present invention the inventors have purified and cloned a novel sphingosine kinase molecule.

#### SUMMARY OF THE INVENTION

Sequence Identity Numbers (<400>X) for the nucleotide and amino acid sequences referred to in the specification are defined following the Examples.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention provides an isolated nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding or complementary to a sequence encoding a novel sphingosine kinase protein or a derivative or mimetic of said sphingosine kinase protein.

5

Another aspect of the present invention provides an isolated nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding or complementary to a sequence encoding a human sphingosine kinase protein or a derivative or mimetic of said sphingosine kinase protein.

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Yet another aspect of the present invention provides a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or having at least about 45% or greater similarity to at least 10 contiguous amino acids in <400>2.

15

Still another aspect of the present invention contemplates a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence substantially as set forth in <400>1 or a derivative thereof or capable of hybridising to <400>1 under low stringency conditions.

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Still yet another aspect of the present invention contemplates a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence substantially as set forth in <400>1 or a derivative thereof, or capable of hybridising to <400>1 under low stringency conditions and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in <400>2 or a sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2.

25

A further aspect of the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in <400>3.

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Another further aspect of the present invention contemplates a genomic nucleic acid molecule or derivative or analogue thereof capable of hybridising to <400>1 or a derivative thereof under low stringency conditions at 42°C.

- 5 Still another further aspect of the present invention provides a cDNA sequence comprising a sequence of nucleotides as set forth in <400>1 or a derivative or analogue thereof including a nucleic acid nucleotide sequence having similarity to <400>1 or a derivative or analogue thereof encoding or complementing a human sphingosine kinase gene or a derivative or analogue thereof.

- Yet another further aspect of the present invention provides an amino acid sequence set forth in  
10 <400>2 or a derivative, analogue or chemical equivalent or mimetic thereof as defined above or a derivative or mimetic having an amino acid sequence of at least about 45% similarity to at least 10 contiguous amino acids in the amino acid sequence as set forth in <400>2 or a derivative or analogue or mimetic thereof.

- 15 Still yet another further aspect of the present invention is directed to an isolated protein selected from the list consisting of:

- (i) A novel sphingosine kinase protein or a derivative, analogue, chemical equivalent or mimetic thereof.
- (ii) A human sphingosine kinase protein or a derivative, analogue, chemical equivalent or mimetic thereof.
- (iii) A protein having an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or a sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2 or a derivative, analogue, chemical equivalent or mimetic of said protein.
- (iv) A protein encoded by a nucleotide sequence substantially as set forth in <400>1 or a derivative or analogue thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2 or a

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derivative, analogue, chemical equivalent or mimetic of said protein.

- (v) A protein encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in <400>1 or a derivative or analogue thereof under low stringency conditions and which encodes an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or an amino acid sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2. <400>1

- (vi) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a homodimeric form.

- (vii) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a heterodimeric form.

Another aspect of the present invention contemplates a method of modulating activity of sphingosine kinase in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease sphingosine kinase activity.

Still another aspect of the present invention contemplates a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding sphingosine kinase or sufficient to modulate the activity of sphingosine kinase.

Yet another aspect of the present invention contemplates a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of sphingosine kinase or *sphingosine kinase* or a derivative or analogue or mimetic of said protein.

Still yet another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of *sphingosine kinase* or sufficient to modulate the activity of sphingosine kinase wherein said modulation results in modulation of

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cellular functional activity.

A further aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of sphingosine kinase or  
5 *sphingosine kinase* for a time and under conditions sufficient to modulate cellular functional activity.

Yet another further aspect of the present invention relates to the use of an agent capable of modulating the expression of *sphingosine kinase* or modulating the activity of sphingosine kinase  
10 in the manufacture of a medicament for the modulation of cellular functional activity.

A further aspect of the present invention relates to the use of sphingosine kinase or *sphingosine kinase* in the manufacture of a medicament for the modulation of cellular functional activity.

15 Still yet another aspect of the present invention relates to agents for use in modulating *sphingosine kinase* expression or sphingosine kinase activity wherein said modulation results in modulation of cellular functional activity.

Another aspect of the present invention relates to sphingosine kinase or *sphingosine kinase* for  
20 use in modulating cellular functional activity.

In a related aspect of the present invention, the mammal undergoing treatment may be a human or an animal in need of therapeutic or prophylactic treatment.

25 In yet another further aspect the present invention contemplates a pharmaceutical composition comprising *sphingosine kinase*, sphingosine kinase or an agent capable of modulating *sphingosine kinase* expression or sphingosine kinase activity together with one or more pharmaceutically acceptable carriers and/or diluents. *Sphingosine kinase*, sphingosine kinase or said agent are referred to as the active ingredients.

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Yet another aspect of the present invention contemplates a method for detecting sphingosine kinase or *sphingosine kinase* mRNA in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for sphingosine kinase or *sphingosine kinase* mRNA or its derivatives or homologs for a time and under conditions  
 5 sufficient for an antibody-sphingosine kinase or antibody-sphingosine kinase mRNA complex to form, and then detecting said complex.

Yet another further aspect of the present invention relates to a method for modulating the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue by the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. Another aspect of the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. Still yet another aspect of the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. Another aspect of the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue.

Another aspect of the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. In addition, the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. In addition, the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. In addition, the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue. In addition, the present invention relates to a method for the manufacture of a nucleic acid construct for the modulation of the expression of *sphingosine kinase* or *sphingosine kinase* mRNA in a cell or tissue.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

**TABLE 1**  
**Single and three letter amino acid abbreviations**

5	Amino Acid	Three-letter Abbreviation	One-letter Symbol
	Alanine	Ala	A
10	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic acid	Asp	D
	Cysteine	Cys	C
	Glutamine	Gln	Q
15	Glutamic acid	Glu	E
	Glycine	Gly	G
	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
20	Lysine	Lys	K
	Methionine	Met	M
	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
25	Threonine	The	T
	Tryptophan	Trp	W
	Tyrosine	Tyr	Y
	Valine	Val	V
30	Any residue	Xaa	X

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a schematic representation of the 'Sphingomyelin pathway'.

- 5 **Figure 2** is a graphical representation of the ammonium sulphate fractionation of sphingosine kinase from human placenta. Ammonium sulphate fractionation of sphingosine kinase in the soluble fraction of the human placenta homogenate was carried out by the addition of solid ammonium sulphate to the desired concentration with gentle stirring at 4°C. Precipitated proteins were recovered by centrifugation at 20 000 g for 30 min at 4°C, redissolved in Buffer B and desalted by extensive dialysis against Buffer B.

- Figure 3** is a graphical representation of anion exchange chromatography of human placenta sphingosine kinase with Q-Sepharose. The dialysed ammonium sulphate fraction containing sphingosine kinase activity was applied to a Q-Sepharose Fast Flow (Pharmacia) column (50 mm diameter, 250 ml bed volume) pre-equilibrated with Buffer A at a flow rate of 7 ml/min. Sphingosine kinase activity was eluted with a NaCl gradient of 0 to 1M and collected in 10 ml fractions. ◆, sphingosine kinase activity; —, absorbance at 280 nm; - - - - , NaCl concentration. Two peaks of sphingosine kinase activity were detected and designated SKI and SKII in order of their elution from this column.

- 20 **Figure 4** is a graphical representation of calmodulin affinity chromatography of human placenta sphingosine kinase I (SKI) with Calmodulin Sepharose. The Q-Sepharose FF column fractions containing highest SKI activity were combined and CaCl<sub>2</sub> and NaCl added to give final concentrations of 4 mM and 250 mM, respectively. This pooled extract was then applied to a Calmodulin Sepharose (Pharmacia) column (16 mm diameter, 10 ml bed volume) pre-equilibrated with Buffer A containing 2 mM EGTA at a flow rate of 1 ml/min. Protein was eluted initially with Buffer A containing 4 mM EGTA and then with Buffer A containing 4 mM EGTA and 1 M NaCl. ■, sphingosine kinase activity; —, absorbance at 280 nm; - - - - , NaCl concentration.

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**Figure 5** is a graphical representation of anion exchange chromatography of human placenta sphingosine kinase with Mono-Q. The Calmodulin Sepharose fractions containing highest sphingosine kinase activity were pooled, desalted on a Sephadex G-25 column, and applied at a flow rate of 1 ml/min to a Mono-Q (Pharmacia) column (5 mm diameter, 1 ml bed volume) pre-equilibrated with Buffer A. Sphingosine kinase activity was eluted with a NaCl gradient of 0 to 1M in Buffer A. NaCl (to 500 mM) was immediately added to the fractions (1 ml) collected to stabilize enzyme activity. ■, sphingosine kinase activity; —, absorbance at 280 nm; ---, NaCl concentration.

**Figure 6** is a graphical representation of anion exchange chromatography of human placenta sphingosine kinase with ATP and Mono-Q. The Mono-Q fractions containing highest sphingosine kinase activity were pooled and desalted on a Sephadex G-25 column. ATP was then added to the pooled fractions to a final concentration of 1 mM before application at a flow rate of 1 ml/min to a Mono-Q (Pharmacia) column (5 mm diameter, 1 ml bed volume) pre-equilibrated with Buffer A containing 1 mM ATP. Sphingosine kinase activity was eluted with a NaCl gradient of 0 to 1M in Buffer A containing 1 mM ATP. NaCl (to 500 mM) was immediately added to the fractions (1 ml) collected to stabilize enzyme activity. ■, sphingosine kinase activity; —, absorbance at 280 nm; ---, NaCl concentration.

**Figure 7** is a graphical representation of gel filtration chromatography of human placenta sphingosine kinase with Superdex 75. The ATP-Mono-Q fractions containing highest sphingosine kinase activity were pooled and concentrated 10-fold to a final volume of 200 µl and applied at a flow rate of 0.5 ml/min to a Superdex 75 (Pharmacia) column (10 mm diameter, 20 ml bed volume) pre-equilibrated with Buffer A containing 500 mM NaCl. Sphingosine kinase activity was eluted with the same buffer and 0.5 ml fractions collected. ■, sphingosine kinase activity; —, absorbance at 280 nm.

**Figure 8** is a graphical representation of second gel filtration chromatography of human placenta sphingosine kinase with Superdex 75. The Superdex 75 fractions containing highest sphingosine kinase activity were pooled and concentrated 5-fold to a final volume of 200 µl and again applied at a flow rate of 0.5 ml/min to a Superdex 75 (Pharmacia) column (10 mm diameter, 20

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ml bed volume) pre-equilibrated with Buffer A containing 500 mM NaCl. Sphingosine kinase activity was eluted with the same buffer and 0.5 ml fractions collected. ■, sphingosine kinase activity; —, absorbance at 280 nm.

5 **Figure 9** is a photographic representation of SDS-PAGE of purified human placenta sphingosine kinase. The fraction from the second Superdex 75 column containing the highest sphingosine kinase activity was applied to SDS-PAGE with silver staining yielding a single band of 45 kDa.

**Figure 10** is a schematic representation showing coverage of the human sphingosine kinase cDNA sequence by EST sequences. The TGA sequence represents the termination codon of SPHK. Note that the N-terminal sequence and a central region of SPHK coding sequence is not present in the database.

**Figure 11** is a schematic representation of the strategy used to clone sphingosine kinase (SPHK) from HUVEC.

**Figure 12** is a schematic representation of nucleotide (<400>1) and predicted amino acid sequence (<400>2) of SPHK. The SPHK coding region is in capital letters (nucleotides 33-1187). Lower case letters denote untranslated and vector sequences.

20 **Figure 13** is a graphical representation of the activity of HUVEC skase over-expressed in HEK293 cells. HEK293 cells were transiently transfected with the pcDNA3 sphingosine kinase expression construct (see Fig. 11) or vector alone. At 48 hrs post-transfection cells were lysed in Buffer B and sphingosine kinase activity measured as described in Table 1.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the purification and cloning of a novel sphingosine kinase molecule. The identification of this novel molecule permits the identification and rational design of a range of products for use in therapy, diagnosis and antibody generation, for example for use in signal transduction. These therapeutic molecules may also act as either antagonists or agonists of sphingosine kinase function and will be useful, *inter alia*, in the modulation of cellular activation in the treatment of disease conditions characterised by unwanted cellular activity.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding or complementary to a sequence encoding a novel sphingosine kinase protein or a derivative or mimetic of said sphingosine kinase protein.

Reference to "sphingosine kinase" should be understood as a reference to the molecule which is, *inter alia*, involved in the generation of sphingosine-1-phosphate during the activation of the sphingosine kinase signalling pathway. Reference to "*sphingosine kinase*" in italicised text should be understood as a reference to the sphingosine kinase nucleic acid molecule. Reference to "sphingosine kinase" in non-italicised text should be understood as a reference to the sphingosine kinase protein molecule.

More particularly, the present invention provides an isolated nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding or complementary to a sequence encoding a human sphingosine kinase protein or a derivative or mimetic of said sphingosine kinase protein.

In a preferred embodiment, the present invention provides a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or having at least about 45% or greater

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similarity to at least 10 contiguous amino acids in <400>2.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. The percentage similarity may be greater than 50% such as at least 70% or at least 80% or at least 90% or at least 95% or higher.

Another aspect of the present invention contemplates a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence substantially as set forth in <400>1 or a derivative thereof, or capable of hybridising to <400>1 under low stringency conditions.

Reference herein to a low stringency includes and encompasses from at least about 0% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions. Stringency may be measured using a range of temperature such as from about 40°C to about 65°C. Particularly useful stringency conditions are at 42°C. In general, washing is carried out at  $T_m = 69.3 + 0.41 (G + C) \% [19] = -12^\circ\text{C}$ . However, the  $T_m$  of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatched based pairs (Bonner *et al.* (1993) *J.Mol.Biol.* 81:123).

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Preferably, the present invention contemplates a nucleic acid molecule or derivative or analogue thereof comprising a nucleotide sequence substantially as set forth in <400>1 or a derivative thereof or capable of hybridising to <400>1 under low stringency conditions and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in <400>2 or a  
 5 sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2.

More particularly, the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence of nucleotides substantially as set forth in <400>1

10 The nucleic acid molecule according to this aspect of the present invention corresponds herein to human *sphingosine kinase*. Without limiting the present invention to any one theory or mode of action, the protein encoded by *sphingosine kinase* is a key element in the functioning of the sphingosine kinase signalling pathway. Sphingosine kinase acts to facilitate the generation of the second messenger, sphingosine-1-phosphate, and may be activated by:

15

- (a) post-translational modifications such as phosphorylation or proteolytic cleavage;
- (b) protein-protein interactions such as dimerisation, and G protein coupled receptor mediated interactions;
- (c) translocational events where the enzyme is targeted to an environment that increases catalytic activity or allows access to its substrate.

20 The expression product of the human *sphingosine kinase* nucleic acid molecule is human sphingosine kinase. Sphingosine kinase is defined by the amino acid sequence set forth in <400>2. The cDNA sequence for sphingosine kinase is defined by the nucleotide sequence set forth in <400>1. The nucleic acid molecule encoding sphingosine kinase is preferably a sequence of deoxyribonucleic acids such as a cDNA sequence or a genomic sequence. A genomic sequence may also comprise exons and introns. A genomic sequence may also include a  
 30 promoter region or other regulatory regions.



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Another aspect of the present invention contemplates a genomic nucleic acid molecule or derivative thereof capable of hybridising to <400>1 or a derivative thereof under low stringency conditions at 42°C.

- 5 Reference herein to sphingosine kinase and *sphingosine kinase* should be understood as a reference to all forms of human sphingosine kinase and *sphingosine kinase*, respectfully, including, for example, any peptide and cDNA isoforms which arise from alternative splicing of *sphingosine kinase* mRNA or mutants or polymorphic variants of *sphingosine kinase* or sphingosine kinase. To the extent that it is not specified, reference herein to sphingosine kinase  
 10 and *sphingosine kinase* includes reference to derivatives, analogues, chemical equivalents and mimetics thereof.

The protein and/or gene is preferably from the human. However, the protein and/or gene may also be isolated from other animal or non-animal species.

15

Derivatives include fragments, parts, portions, mutants, variants, and mimetics from natural, synthetic or recombinant sources including fusion proteins. Parts or fragments include, for example, active regions of sphingosine kinase. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or  
 20 carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are  
 25 those in which at least one residue in the sequence has been removed and a different residue inserted in its place. An example of substitutional amino acid variants are conservative amino acid substitutions. Conservative amino acid substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and  
 30 phenylalanine and tyrosine. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

Chemical and functional equivalents of *sphingosine kinase* or sphingosine kinase should be understood as molecules exhibiting any one or more of the functional activities of *sphingosine kinase* or sphingosine kinase and may be derived from any source such as being chemically synthesized or identified via screening processes such as natural product screening.

5

The derivatives of sphingosine kinase include fragments having particular epitopes or parts of the entire sphingosine kinase protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules.

10 Analogues of sphingosine kinase contemplated herein include, but are not limited to, modifications to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

15 Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

20

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with  $\text{NaBH}_4$ ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2,4,6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with  $\text{NaBH}_4$ .

30 The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 2.

25 with 2, 4,

hydroxy

hydroxy

line

out

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 $\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
10 aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgln
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine 2-H	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15 D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20 D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
25 D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30 D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgab

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D- $\alpha$ -methylalanine	Dmala	$\alpha$ -methylcyclohexylalanine	Mchexa
D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
5 D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	INaeg
D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	INorm
D- $\alpha$ -methylisoleucine	Dmile	N-amino- $\alpha$ -methylbutyrate	INmaabu
D- $\alpha$ -methyllaucine	Dmleu	$\alpha$ -naphthylalanine	INap
10 D- $\alpha$ -methyllysine	Dmlys	N-benzylglycine	INphe
D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	INglu
D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	INasn
D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	INglu
D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	INasp
15 D- $\alpha$ -methylserine	Dmiser	N-cyclobutylglycine	INcbut
D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	INchep
D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	INchex
D- $\alpha$ -methyltyrosine	Dmty	N-cyclodecylglycine	INcdec
D- $\alpha$ -methylvaline	Dmval	N-cylcododecylglycine	INcdod
20 D-N-methylalanine	Dnmala	N-cyclooctylglycine	INcoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	INcpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	INcund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	INbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	INbhe
25 D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	INarg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	INthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	INser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	INhis
D-N-methyllaucine	Dnmleu	N-(3-indolylyethyl)glycine	INhtrp
30 D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	INmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	INmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	INmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	INmphe

N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
5 D-N-methyltyrosine	Dnmtyr	N-methyl- $\alpha$ -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
10 L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	NMala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylasparagine	NMasn
L- $\alpha$ -methylaspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	NMtbug
L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metgar
L- $\alpha$ -methylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
15 L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomophenylalanine	Mhphe
L- $\alpha$ -methyloisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- $\alpha$ -methylleucine	Mleu	L- $\alpha$ -methyllysine	Mlys
L- $\alpha$ -methylmethionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
20 L- $\alpha$ -methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
L- $\alpha$ -methylserine	Mser	L- $\alpha$ -methylthreonine	Mthr
L- $\alpha$ -methyltryptophan	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr
L- $\alpha$ -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
25 carbamylmethyl)glycine		carbamylmethyl)glycine	
1-carboxy-1-(2,2-diphenyl-Nmbc			
ethylamino)cyclopropane			

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having  $(CH_2)_n$  spacer groups with  $n=1$  to  $n=6$ , glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

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The nucleic acid molecule of the present invention is preferably in isolated form or ligated to a vector, such as an expression vector. By "isolated" is meant a nucleic acid molecule having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject nucleic acid molecule, preferably at least  
 5 about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject nucleic acid molecule relative to other components as determined by molecular weight, encoding activity, nucleotide sequence, base composition or other convenient means. The nucleic acid molecule of the present invention may also be considered, in a  
 10 preferred embodiment, to be biologically pure.

The term "protein" should be understood to encompass peptides, polypeptides and proteins. The protein may be glycosylated or unglycosylated and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the protein such as amino acids,  
 15 lipids, carbohydrates or other peptides, polypeptides or proteins. Reference hereinafter to a "protein" includes a protein comprising a sequence of amino acids as well as a protein associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins.

20 In a particularly preferred embodiment, the nucleotide sequence corresponding to *sphingosine kinase* is a cDNA sequence comprising a sequence of nucleotides as set forth in <400>1 or a derivative or analogue thereof including a nucleotide sequence having similarity to <400>1.

A derivative of a nucleic acid molecule of the present invention also includes a nucleic acid  
 25 molecule capable of hybridising to a nucleotide sequence as set forth in <400>1 under low stringency conditions. Preferably, low stringency is at 42°C.

The nucleic acid molecule may be ligated to an expression vector capable of expression in a prokaryotic cell (e.g. *E.coli*) or a eukaryotic cell (e.g. yeast cells, fungal cells, insect cells,  
 30 mammalian cells or plant cells). The nucleic acid molecule may be ligated or fused or otherwise associated with a nucleic acid molecule encoding another entity such as, for example, a signal

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peptide. It may also comprise additional nucleotide sequence information fused, linked or otherwise associated with it either at the 3' or 5' terminal portions or at both the 3' and 5' terminal portions. The nucleic acid molecule may also be part of a vector, such as an expression vector. The latter embodiment facilitates production of recombinant forms of sphingosine kinase which forms are encompassed by the present invention.

The present invention extends to the expression product of the nucleic acid molecules as hereinbefore defined.

10 The expression product is sphingosine kinase having an amino acid sequence set forth in <400>2 or is a derivative, analogue or chemical equivalent or mimetic thereof as defined above or is a derivative or mimetic having an amino acid sequence of at least about 45% similarity to at least 10 contiguous amino acids in the amino acid sequence as set forth in <400>2 or a derivative or mimetic thereof.

15 Another aspect of the present invention is directed to an isolated protein selected from the list consisting of:

- 20 (i) A novel sphingosine kinase protein or a derivative, analogue, chemical equivalent or mimetic thereof.
- (ii) A human sphingosine kinase protein or a derivative, analogue, chemical equivalent or mimetic thereof.
- 25 (iii) A protein having an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or a sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2 or a derivative, analogue, chemical equivalent or mimetic of said protein.

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- (iv) A protein encoded by a nucleotide sequence substantially as set forth in <400>1 or a derivative or analogue thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to at least 10 contiguous amino acids in <400>2 or a derivative, analogue, chemical equivalent or mimetic of said protein.
- 5 (v) A protein encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in <400>1 or a derivative or analogue thereof under low stringency conditions and which encodes an amino acid sequence substantially as set forth in <400>2 or a derivative or mimetic thereof or an amino acid sequence having at  
10 least about 45% similarity to at least 10 contiguous amino acids in <400>2.
- (vi) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a homodimeric form.
- (vii) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a heterodimeric  
15 form.

The protein of the present invention is preferably in isolated form. By "isolated" is meant a protein having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject protein, preferably at least  
20 about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject protein relative to other components as determined by molecular weight, amino acid sequence or other convenient means. The protein of the present invention may also be considered, in a preferred embodiment, to be biologically pure.

25 The sphingosine kinase of the present invention may be in multimeric form meaning that two or more molecules are associated together. Where the same sphingosine kinase molecules are associated together, the complex is a homomultimer. An example of a homomultimer is a homodimer. Where at least one sphingosine kinase is associated with at least one non-  
30 sphingosine kinase molecule, then the complex is a heteromultimer such as a heterodimer.

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The ability to produce recombinant sphingosine kinase permits the large scale production of sphingosine kinase for commercial use. The sphingosine kinase may need to be produced as part of a large peptide, polypeptide or protein which may be used as is or may first need to be processed in order to remove the extraneous proteinaceous sequences. Such processing  
5 includes digestion with proteases, peptidases and amidases or a range of chemical, electrochemical, sonic or mechanical disruption techniques.

Notwithstanding that the present invention encompasses recombinant proteins, chemical  
10 synthetic techniques are also preferred in synthesis of sphingosine kinase.

Sphingosine kinase according to the present invention is conveniently synthesised based on molecules isolated from the human. Isolation of the human molecules may be accomplished by  
15 any suitable means such as by chromatographic separation, for example using CM-cellulose ion exchange chromatography followed by Sephadex (e.g. G-50 column) filtration. Many other techniques are available including HPLC, PAGE amongst others.

Sphingosine kinase may be synthesised by solid phase synthesis using F-moc chemistry as described by Carpino *et al.* (1991). Sphingosine kinase and fragments thereof may also be synthesised by alternative chemistries including, but not limited to, t-Boc chemistry as described  
20 in Stewart *et al.* (1985) or by classical methods of liquid phase peptide synthesis.

Without limiting the theory or mode of action of the present invention, sphingosine kinase is a key regulatory enzyme in the activity of the sphingosine kinase signalling pathway. By "sphingosine kinase signalling pathway" is meant a signalling pathway which utilises one or both  
25 of sphingosine kinase and/or sphingosine-1-phosphate. It is thought that a sphingosine kinase signalling pathway cascade which results in adhesion molecule expression may take the form of:

- (i) the generation of ceramide from sphingomyelin via *S.Mase* activity, said  
30 ceramide being converted to sphingosine;

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(ii) sphingosine-1-phosphate (referred to hereinafter as "Sph-1-P") generation by stimulation of sphingosine kinase; and

(iii) the activation of MEK/ERK and nuclear translocation of NF- $\kappa$ B downstream from Sph-1-P generation.

The sphingosine kinase signalling pathway is known to regulate cellular activities such as those which lead to inflammation, apoptosis and cell proliferation. For example, upregulation of the production of inflammatory mediators such as cytokines, chemokines, eNOS and upregulation of adhesion molecule expression. Said upregulation may be induced by a number of stimuli including, for example, inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1), endotoxin, oxidised or modified lipids, radiation or tissue injury.

The cloning and sequencing of this gene and its expression product now provides additional molecules for use in the prophylactic and therapeutic treatment of diseases characterised by unwanted cellular activity, which activity is either directly or indirectly modulated via the activity of the sphingosine kinase signalling pathway. Examples of diseases involving unwanted sphingosine kinase regulated cellular activity include rheumatoid arthritis, asthma, atherosclerosis, meningitis, multiple sclerosis and septic shock. Accordingly, the present invention contemplates therapeutic and prophylactic uses of sphingosine kinase amino acid and nucleic acid molecules, in addition to sphingosine kinase agonistic and antagonistic agents, for the regulation of cellular functional activity, such as for example, regulation of inflammation.

The present invention contemplates, therefore, a method for modulating expression of *sphingosine kinase* in a subject, said method comprising contacting the *sphingosine kinase* gene with an effective amount of an agent for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *sphingosine kinase*. For example, *sphingosine kinase* antisense sequences such as oligonucleotides may be introduced into a cell to down-regulate one or more specific functional activities of that cell. Conversely, a nucleic acid molecule encoding sphingosine kinase or a derivative thereof may be introduced to up-regulate one or more specific functional activities of any cell not expressing the

endogenous *sphingosine kinase* gene.

Another aspect of the present invention contemplates a method of modulating activity of sphingosine kinase in a mammal, said method comprising administering to said mammal a  
5 modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease sphingosine kinase activity.

Modulation of said activity by the administration of an agent to a mammal can be achieved by one of several techniques, including but in no way limited to introducing into said mammal a  
10 proteinaceous or non-proteinaceous molecule which:

- (i) modulates expression of *sphingosine kinase*;
- (ii) functions as an antagonist of sphingosine kinase;
- 15 (iii) functions as an agonist of sphingosine kinase.

Said proteinaceous molecule may be derived from natural or recombinant sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous  
20 molecule may be, for example, a nucleic acid molecule or may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. The present invention contemplates chemical analogs of sphingosine kinase or small molecules capable of acting as agonists or antagonists of sphingosine kinase. Chemical agonists may not necessarily be derived from sphingosine kinase but may share certain conformational similarities.  
25 Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of sphingosine kinase. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing sphingosine kinase from carrying out its normal biological functions. Antagonists include monoclonal antibodies specific for sphingosine kinase, or parts of sphingosine kinase, and antisense nucleic acids which prevent transcription or translation of  
30 *sphingosine kinase* genes or mRNA in mammalian cells. Modulation of *sphingosine kinase* expression may also be achieved utilising antigens, RNA, ribosomes, DNazymes, RNA

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aptamers or antibodies.

Said proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of *sphingosine kinase* or the activity of sphingosine kinase. Said molecule acts directly if it associates with *sphingosine kinase* or sphingosine kinase to modulate the expression or activity of *sphingosine kinase* or sphingosine kinase. Said molecule acts indirectly if it associates with a molecule other than *sphingosine kinase* or sphingosine kinase which other molecule either directly or indirectly modulates the expression or activity of *sphingosine kinase* or sphingosine kinase. Accordingly, the method of the present invention encompasses the regulation of *sphingosine kinase* or sphingosine kinase expression or activity via the induction of a cascade of regulatory steps which lead to the regulation of *sphingosine kinase* or sphingosine kinase expression or activity.

Another aspect of the present invention contemplates a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding sphingosine kinase or sufficient to modulate the activity of sphingosine kinase.

Yet another aspect of the present invention contemplates a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of sphingosine kinase or *sphingosine kinase*.

The sphingosine kinase, *sphingosine kinase* or agent used may also be linked to a targeting means such as a monoclonal antibody, which provides specific delivery of the sphingosine kinase, *sphingosine kinase* or agent to the target cells.

In a preferred embodiment of the present invention, the sphingosine kinase, *sphingosine kinase* or agent used in the method is linked to an antibody specific for said target cells to enable specific delivery to these cells.

Reference to "modulating cellular functional activity" is a reference to up-regulating, down-regulating or otherwise altering any one or more of the activities which a cell is capable of performing such as, but not limited to, one or more of chemokine production, cytokine production, nitric oxide synthetase, adhesion molecule expression and production of other inflammatory modulators.

Administration of the sphingosine kinase, *sphingosine kinase* or agent, in the form of a pharmaceutical composition, may be performed by any convenient means. Sphingosine kinase, *sphingosine kinase* or agent of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the sphingosine kinase, *sphingosine kinase* or agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of sphingosine kinase or agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The sphingosine kinase or agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intranasal, intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of sphingosine kinase or agent, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

A further aspect of the present invention relates to the use of the invention in relation to mammalian disease conditions. For example, the present invention is particularly useful, but in no way limited to, use in inflammatory diseases.

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Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of *sphingosine kinase* or sufficient to modulate the activity of sphingosine kinase wherein said modulation results in  
5 modulation of cellular functional activity.

In another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of sphingosine kinase or *sphingosine kinase* for a time and under conditions sufficient to modulate cellular functional  
10 activity.

Yet another aspect of the present invention relates to the use of an agent capable of modulating the expression of *sphingosine kinase* or modulating the activity of sphingosine kinase in the manufacture of a medicament for the modulation of cellular functional activity.  
15

A further aspect of the present invention relates to the use of sphingosine kinase or *sphingosine kinase* in the manufacture of a medicament for the modulation of cellular functional activity.

Still yet another aspect of the present invention relates to agents for use in modulating  
20 *sphingosine kinase* expression or sphingosine kinase activity wherein said modulation results in modulation of cellular functional activity.

Another aspect of the present invention relates to sphingosine kinase or *sphingosine kinase* for use in modulating cellular functional activity.  
25

In a related aspect of the present invention, the mammal undergoing treatment may be a human or an animal in need of therapeutic or prophylactic treatment.

In yet another further aspect the present invention contemplates a pharmaceutical composition  
30 comprising *sphingosine kinase*, sphingosine kinase or an agent capable of modulating *sphingosine kinase* expression or sphingosine kinase activity together with one or more

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pharmaceutically acceptable carriers and/or diluents. *Sphingosine kinase*, sphingosine kinase or said agent are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where  
5 water soluble) and sterile powders for the extemporaneous preparation of sterile injectable  
solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that  
easy syringability exists. It must be stable under the conditions of manufacture and storage and  
must be preserved against the contaminating action of microorganisms such as bacteria and  
fungi. The carrier can be a solvent or dispersion medium containing, for example, water,  
10 ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the  
like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for  
example, by the use of a coating such as lecithin, by the maintenance of the required particle size  
in the case of dispersion and by the use of surfactants. The prevention of the action of  
microorganisms can be brought about by various antibacterial and antifungal agents, for  
15 example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases,  
it will be preferable to include isotonic agents, for example, sugars or sodium chloride.  
Prolonged absorption of the injectable compositions can be brought about by the use in the  
compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

20 Sterile injectable solutions are prepared by incorporating the active compounds in the required  
amount in the appropriate solvent with various of the other ingredients enumerated above, as  
required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating  
the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion  
medium and the required other ingredients from those enumerated above. In the case of sterile  
25 powders for the preparation of sterile injectable solutions, the preferred methods of preparation  
are vacuum drying and the freeze-drying technique which yield a powder of the active  
ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When *sphingosine kinase*, sphingosine kinase and sphingosine kinase modulators are suitably  
30 protected they may be orally administered, for example, with an inert diluent or with an  
assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they



may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1  $\mu$ g and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active

ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage: Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5  $\mu$ g to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5  $\mu$ g to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of expressing *sphingosine kinase*, modulating *sphingosine kinase* expression or *sphingosine kinase* activity. The vector may, for example, be a viral vector.

*Sphingosine kinase* can also be utilised to create gene knockout models in either cells or animals, which knocked out gene is the sphingosine kinase gene expressed by said cells or animals. Accordingly in another aspect the present invention should be understood to extend to methods of creating sphingosine kinase gene cell or animal knockout models wherein sphingosine kinase has been utilised to facilitate knocking out of the endogenous sphingosine

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kinase gene of said cell or animal, and to the knockout models produced therefrom.

Still another aspect of the present invention is directed to antibodies to sphingosine kinase including catalytic antibodies. Such antibodies may be monoclonal or polyclonal and may be  
5 selected from naturally occurring antibodies to sphingosine kinase or may be specifically raised to sphingosine kinase. In the case of the latter, sphingosine kinase may first need to be associated with a carrier molecule. The antibodies and/or recombinant sphingosine kinase of the present invention are particularly useful as therapeutic or diagnostic agents. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention  
10 extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and may also be used as a diagnostic tool, for example, for monitoring the program of a therapeutic regime.  
15 For example, sphingosine kinase can be used to screen for naturally occurring antibodies to sphingosine kinase. These may occur, for example, in some inflammatory disorders.

For example, specific antibodies can be used to screen for sphingosine kinase proteins. The latter would be important, for example, as a means for screening for levels of sphingosine kinase  
20 in a cell extract or other biological fluid or purifying sphingosine kinase made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays, ELISA and flow cytometry.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal  
25 or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of sphingosine kinase.

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- Both polyclonal and monoclonal antibodies are obtainable by immunization with the protein or peptide derivatives and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of
- 5 sphingosine kinase, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoabsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.
- 10 The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and
- 15 Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature* 256: 495-499, 1975; *European Journal of Immunology* 6: 511-519, 1976).

In another aspect of the present invention, the molecules of the present invention are also useful

20 as screening targets for use in applications such as the diagnosis of disorders which are regulated by sphingosine kinase.

Yet another aspect of the present invention contemplates a method for detecting sphingosine kinase or *sphingosine kinase* mRNA in a biological sample from a subject said method

25 comprising contacting said biological sample with an antibody specific for sphingosine kinase or *sphingosine kinase* mRNA or its derivatives or homologs for a time and under conditions sufficient for an antibody-sphingosine kinase or antibody-sphingosine kinase mRNA complex to form, and then detecting said complex.

The presence of sphingosine kinase may be determined in a number of ways such as by Western blotting, ELISA or flow cytometry procedures. Sphingosine kinase mRNA may be detected, for example, by *in situ* hybridization or Northern blotting. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the  
5 traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and  
10 all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added  
15 and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the  
20 forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain sphingosine kinase including cell extract, tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and  
25 respiratory fluid. The sample is, therefore, generally a biological sample comprising biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, a first antibody having specificity for the sphingosine kinase or antigenic parts thereof, is either covalently or passively bound to a solid surface. The  
30 solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports

may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is  
5 then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second  
10 antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter  
15 molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

20 By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

25

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-  
30 galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding

- 37 -

enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and  
5 then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or  
10 inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light  
15 energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest.  
20 Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

CO:

The present invention also contemplates genetic assays such as involving PCR analysis to detect  
25 *sphingosine kinase* or its derivatives.

Further features of the present invention are more fully described in the following non-limiting examples.

- 38 -

**EXAMPLE 1****PURIFICATION OF SPHINGOSINE KINASE FROM HUMAN PLACENTA**

Sphingosine kinase was purified from 1240 g of human placenta (4 placentas). The placentas were diced, washed in Buffer A (50 mM Tris/HCl buffer, pH 7.4 containing 10 % (v/v) glycerol, 0.05 % Triton X-100 and 1 mM dithiothreitol), transferred to 1.5 L of fresh Buffer A containing a protease inhibitor cocktail (Complete™; Boehringer Mannheim) (Buffer B), and homogenised in a Waring blender. The resultant homogenate was stored on ice for 30 min to enhance enzyme extraction, and the soluble fraction of the homogenate then isolated by centrifugation at 20 000 g for 60 min at 4 °C. Sphingosine kinase activity was determined by incubating samples at 37°C for 30 min with sphingosine (50 µM; from a 1 mM stock dissolved in 5% Triton X-100) and [ $\gamma$ -<sup>32</sup>P]ATP (1 mM; 10 µCi/ml) in assay buffer containing 50 mM Tris/HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 % glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 15mM NaF, 0.5 mM 4-deoxypyridoxine in a total volume of 100 µl. Reactions were terminated and sphingosine-1-phosphate extracted by the addition of 0.7 ml of chloroform/methanol/HCl (100:200:1, v/v), followed by vigorous mixing, addition of 0.2 ml chloroform and 0.2 ml 2 M KCl, and phase separation by centrifugation. The labeled sphingosine-1-phosphate in the organic phase was isolated by TLC on Silica Gel 60 with 1-butanol/ethanol/acetic acid/water (8:2:1:2, v/v) and quantitated by phosphorimager. One unit (U) of activity is defined as 1 pmol of sphingosine-1-phosphate formed from sphingosine and ATP per minute.

**EXAMPLE 2****STRATEGY USED TO CLONE SPHINGOSINE KINASE (SPHK) FROM HUVEC**

PCR using SPHK primers derived from EST sequence (spanning central *Sac*II site) were used in combination with T3 and T7 primers to amplify SPHK from a HUVEC cDNA library. This generated two overlapping PCR products of 669 bp and 550 bp. These products represented the 5' and 3' ends respectively of SPHK and were separately cloned into pGEM4Z. A 584 bp *Sac*II fragment from the 5' SPHK PCR clone was sub-cloned in the correct orientation into the *Sac*II site of the 3' SPHK PCR clone, to generate a 1130 bp partial SPHK cDNA clone. To



generate a full length clone encoding SPHK, a 120 bp *EcoRI/StuI* fragment from the 669 bp 5' clone was sub-cloned into the pGEM4Z-1130 bp clone digested with *EcoRI/StuI*. Sequencing the cDNA clone in both directions has verified the integrity of the SPHK cDNA sequence. The DNA sequence is shown in Figure 5. For bacterial expression, the full length SPHK cDNA has been sub-cloned into pGEX4T2. Sub-cloning of the SPHK cDNA into the mammalian expression vector pcDNA3 in both orientations enabled production of sense and antisense mRNA in mammalian cells.

### EXAMPLE 3

#### RESULTS

#### *Purification of sphingosine kinase from human placenta*

Sphingosine kinase has been purified from human placenta. This was achieved using ammonium sulphate precipitation and several chromatographic steps (see Table 1 and Figures 2-8); resulting in over a million-fold purification from the original placenta extract, and giving a single silver-stained protein band after SDS-PAGE (Fig. 9). This is the first sphingosine kinase to be purified to homogeneity from a human source.

#### *Cloning of the human sphingosine kinase*

A human sphingosine kinase cDNA has been generated using primers designed from ESTs (Table 2 and Fig 10) aligned with published murine sphingosine kinase sequence [10]. The cloning strategy is shown in Figure 11. The complete cDNA sequence of the HUVEC sphingosine kinase is shown in Figure 12.

#### *Sphingosine kinase activity in mammalian cells*

Preliminary studies have been performed to confirm the activity of the HUVEC sphingosine kinase. The pcDNA3 sphingosine kinase clone shown in Figure 11 was introduced into human embryonic kidney cells (HEK293) by transient transfection. While only modest levels of endogenous sphingosine kinase activity were present in control cells, cells transfected with the HUVEC sphingosine kinase cDNA clone showed a greater than 50 fold increase in activity (see Figure 13).

- 40 -

5 individually or collectively, and any and all combinations of any two or more of said steps or features.

expression of the  $\pi$ - $\pi^*$  interaction of adjacent units is given by  

$$E_{\pi\pi^*} = -\frac{1}{2} \frac{V_{\pi\pi^*}^2}{\epsilon_{\pi\pi^*}}$$

- 41 -

**Table 1. Summary of purification of sphingosine kinase from human placenta.**

Step	Activity	Protein	Specific	Recovery	Purification
Soluble fraction of homogenate	7943	123600	58	100	
Ammonium sulphate (25-35%)	7723	3527	1966	97	33
Q Sepharose anion exchange	4048	1098	4597	63	79
Calmodulin Sepharose	3197	18.23	$1.75 \times 10^5$	40	$3.0 \times 10^3$
Mono Q anion exchange	1706	2.921	$5.84 \times 10^5$	21.5	$1.0 \times 10^4$
ATP- Mono Q anion exchange	1133	0.419	$2.70 \times 10^6$	14.3	$4.6 \times 10^4$
Superdex 75 gel filtration (1)	867	0.088	$9.87 \times 10^6$	10.9	$1.7 \times 10^5$
Superdex 75 gel filtration (2)	549	0.008	$6.64 \times 10^7$	6.9	$1.1 \times 10^6$

**Table 2. Details of human EST sequences.**

This Table shows the human EST sequences that are homologous to the HUVEC SPHK. The Accession number is the database identification code. The length in basepairs (bp) is the amount of EST sequence available. The clone number is the individual cDNA clone identification from a library where available. The source is the mRNA template from which the cDNA library was generated.

Accession number	Length	Clone No. /Source
AAD31133	271bp	human fetal lung
AA026479	341bp	pregnant uterus, Soares clone 469133
W63556	399bp	senescent fibroblasts, Soares clone IMAGE:326250
AA081152	356bp	Endothelial cell, Stratagene clone IMAGE:549298
AA232791	386bp	Pooled melanocyte, fetal heart, pregnant uterus, Soares clone
AI042283	447bp	senescent fibroblasts, Soares clone IMAGE:1665736
AA232646	394bp	Pooled melanocyte, fetal heart, pregnant uterus, Soares clone
AA639414	412bp	colon tumor, clone IMAGE: 1159486

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3. Spiegel, S., Culliver, O., Edsall, L., Koberna, T., Menzelev, R., C  
T., Z., Van, S., J., et War, F. *Proc. Acad. Sci. USSR*, 1957, 10, 1.

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1

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235

240

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His Trp Thr Val Val Pro Asp Glu Asp Phe Val Leu Val Leu Ala Leu

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255

260

ctg cac tgc cac ctg ggc agt gag atg ttt gct gca ccc atg ggc cgc 869

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265

270

275



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35 40 45

Phe Thr Leu Met Leu Thr Glu Arg Arg Asn His Ala Arg Glu Leu Val

50 55 60

Arg Ser Glu Glu Leu Gly Arg Trp Asp Ala Leu Val Val Met Ser Gly

65 70 75 80

Asp Gly Leu Met His Glu Val Val Asn Gly Leu Met Glu Arg Pro Asp

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Trp Glu Thr Ala Ile Gln Lys Pro Leu Cys Ser Leu Pro Ala Gly Ser

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DATED this 13th day of May, 1999.

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by its Patent Attorneys  
DAVIES COLLISON CAVE

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Dr. J. B. ...

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As the value of the

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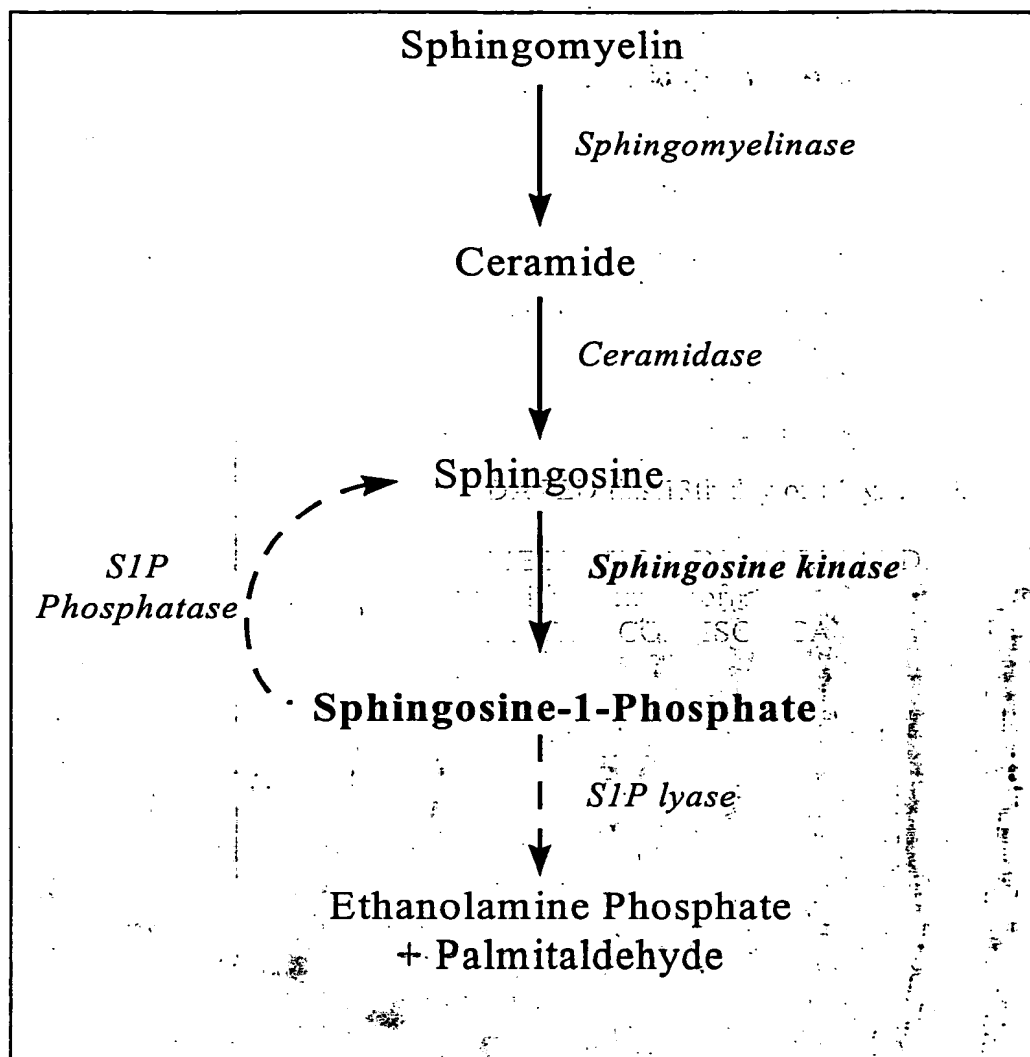
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**Figure 1.**



**Figure 2. Ammonium sulphate precipitation**

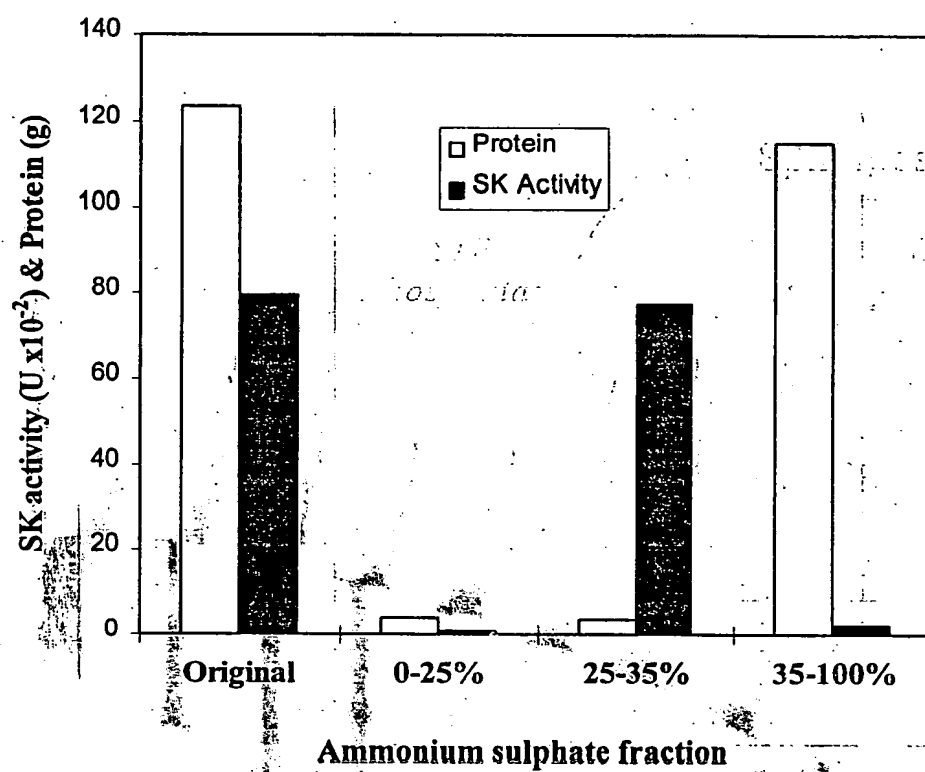


Figure 3. Q Sepharose purification of placenta SK

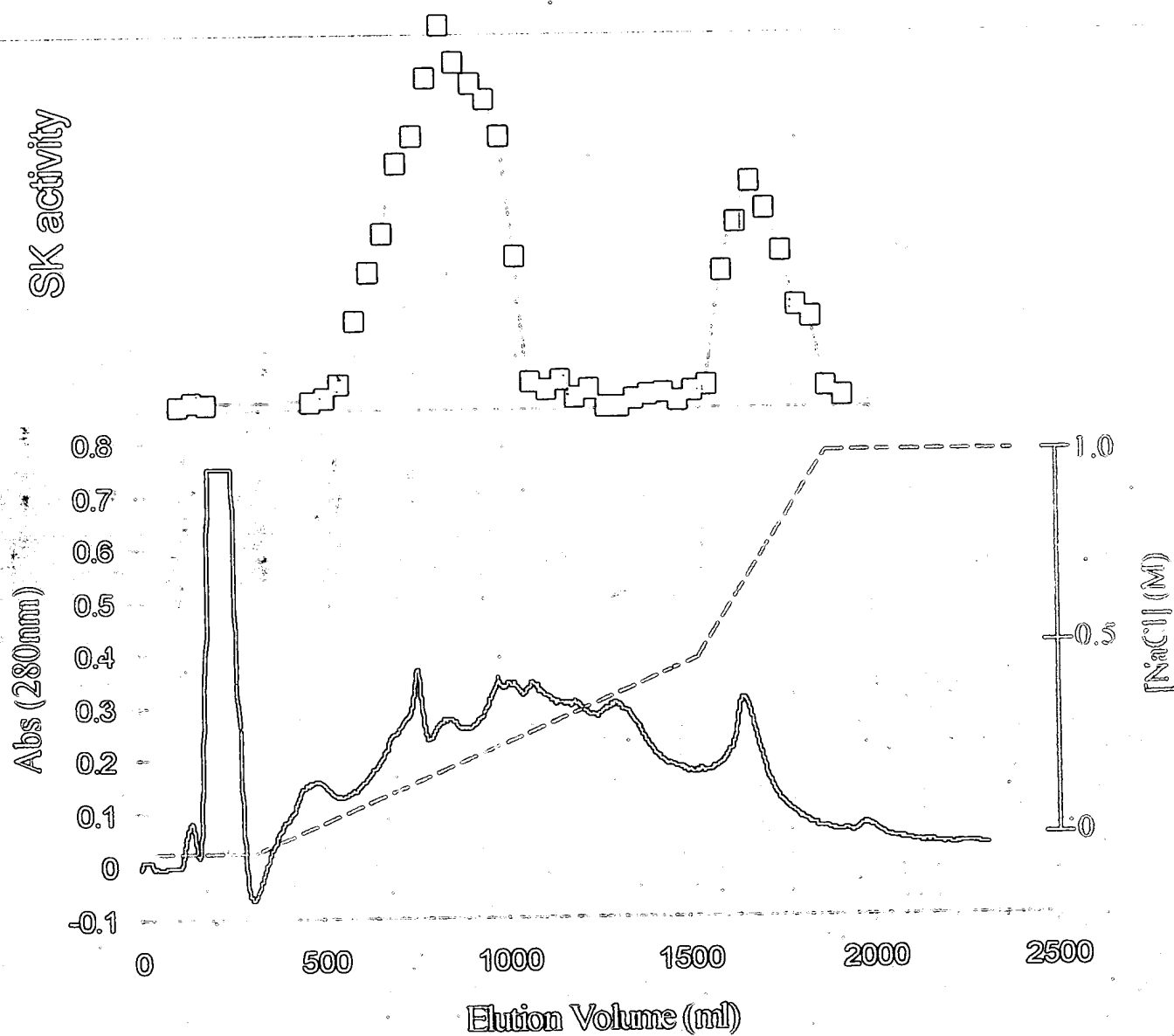


Figure 4. Calmodulin Sepharose affinity purification of human placenta SK

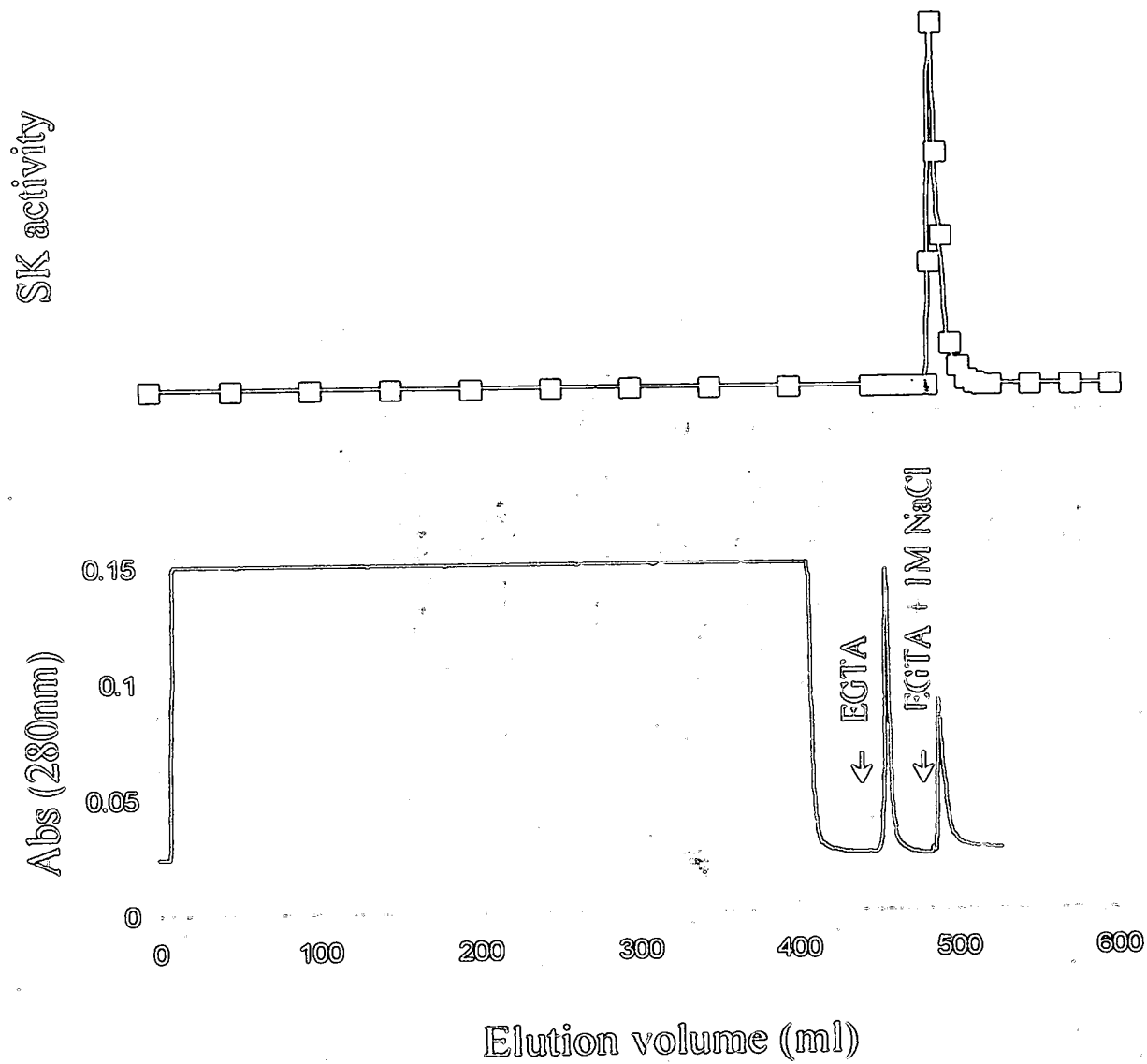




Figure 5. Mono Q anion exchange purification of human placenta SKI

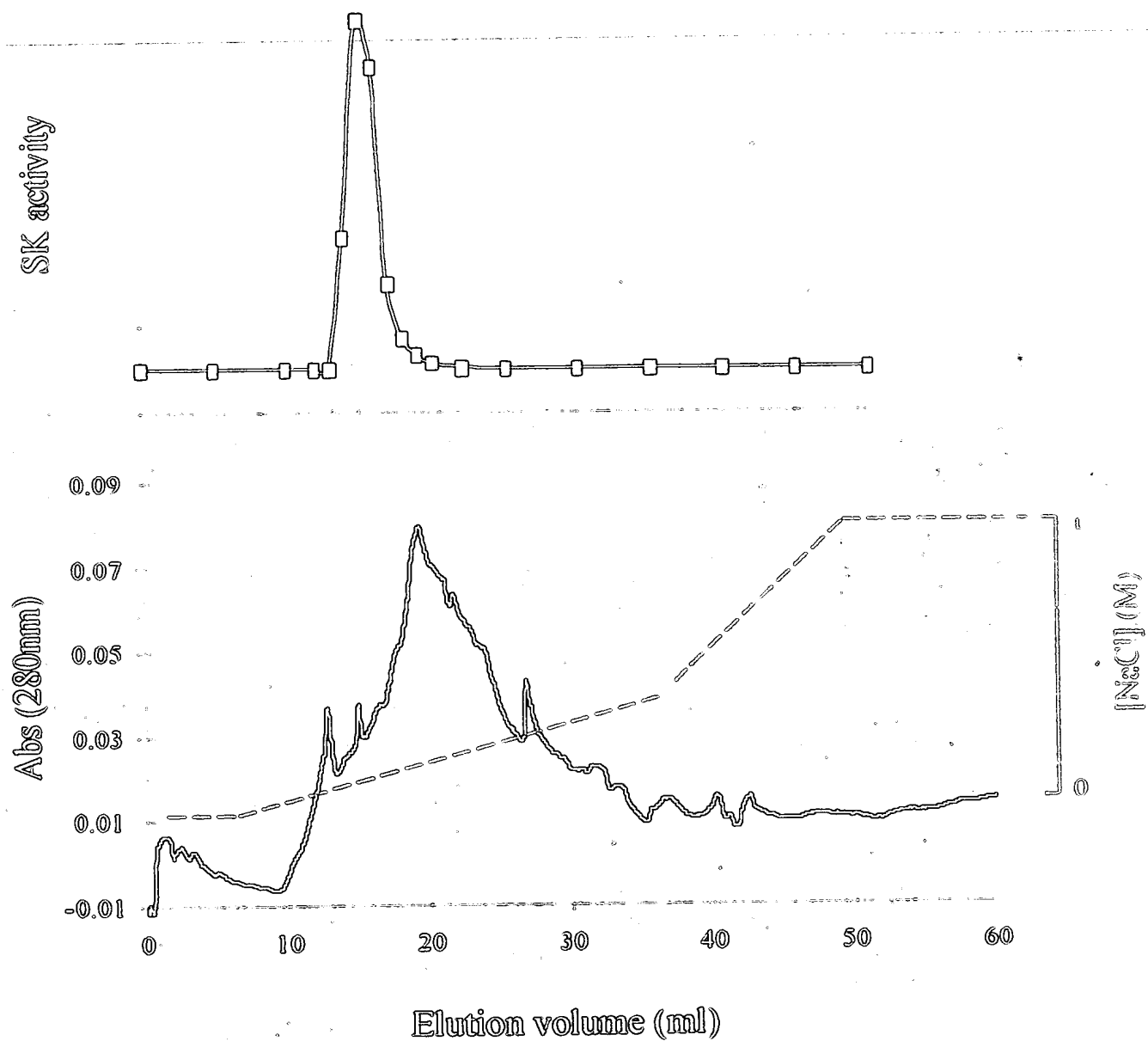


Figure 6. ATP-Mono Q anion exchange chromatography of SK

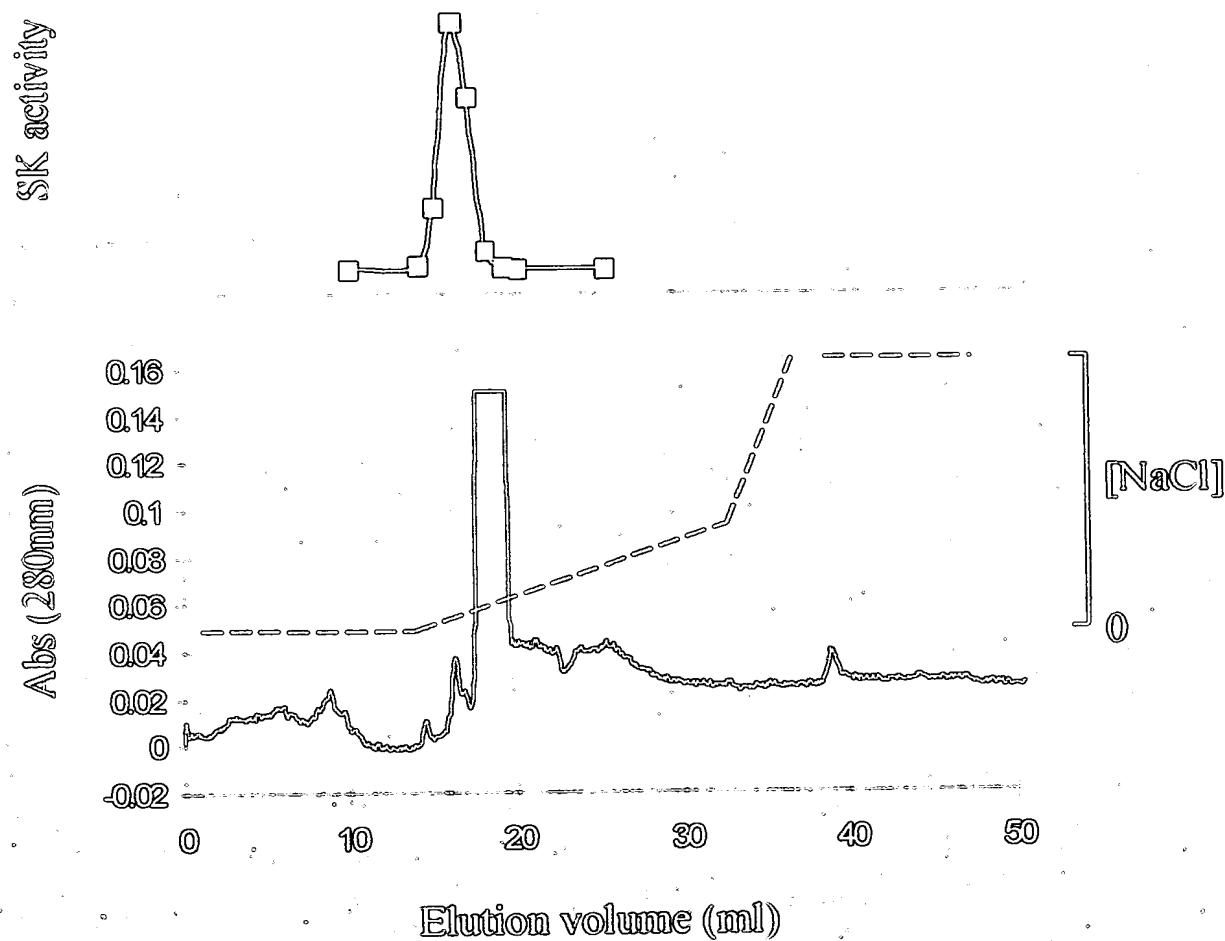


Figure 7. Superdex 75 gel filtration purification of human placenta SKI

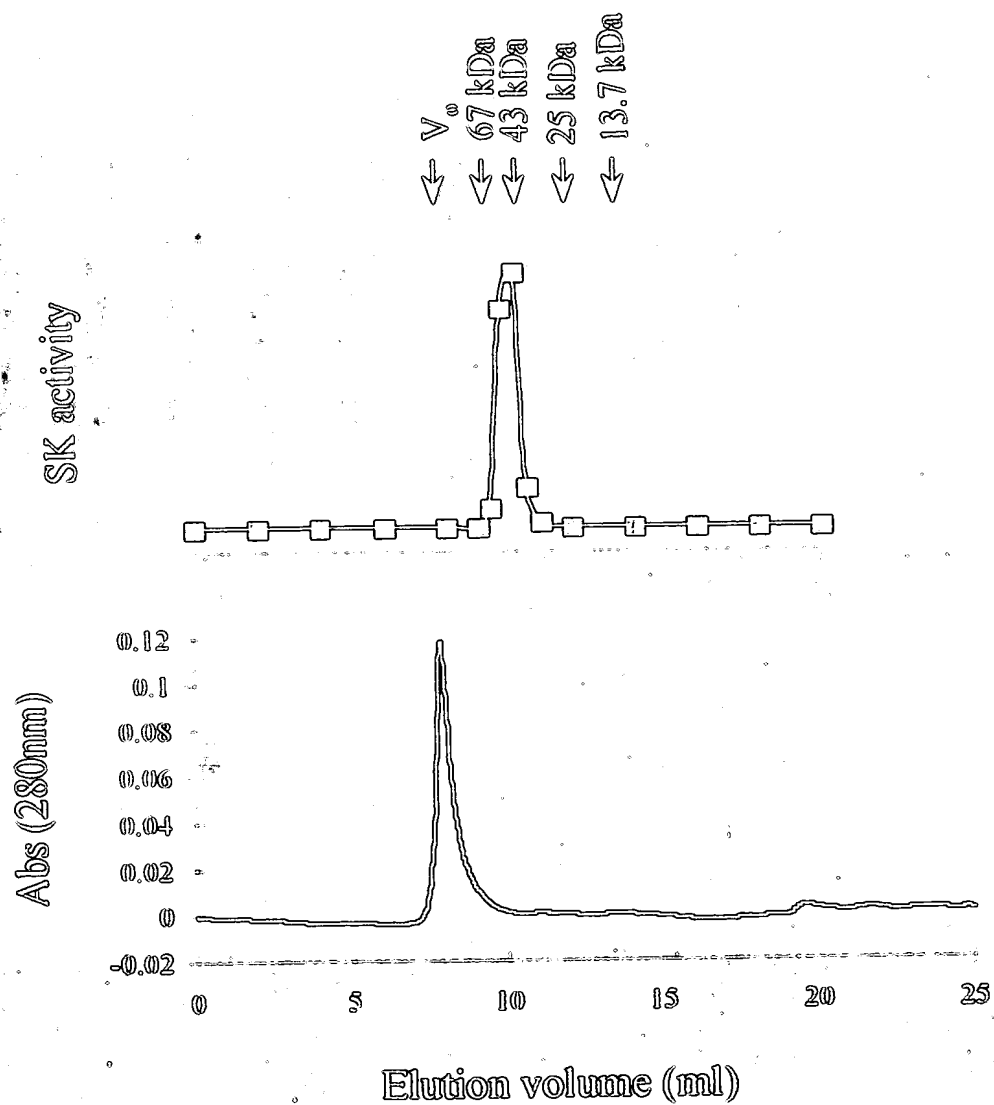
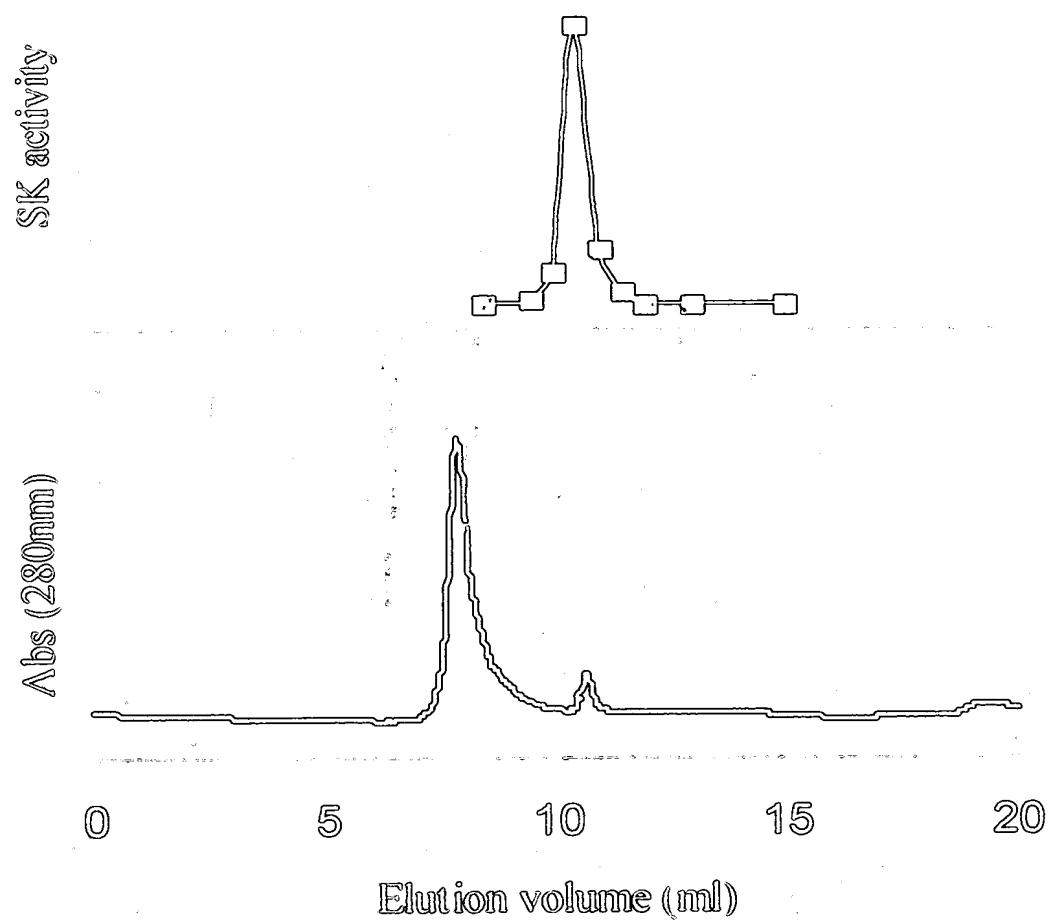
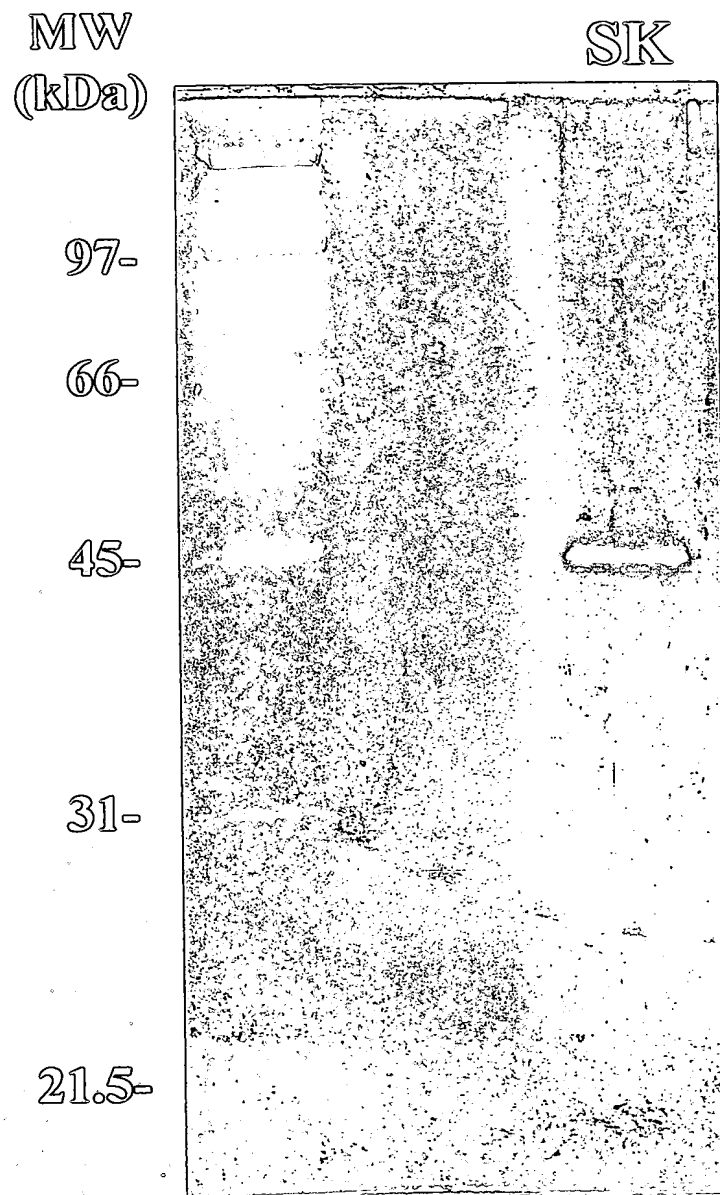


Figure 8. Superdex 75 gel filtration (2) purification of human placenta SK



c

Figure 9. Silver-stained SDS-PAGE gel of purified human placenta SK



**Figure 10.**

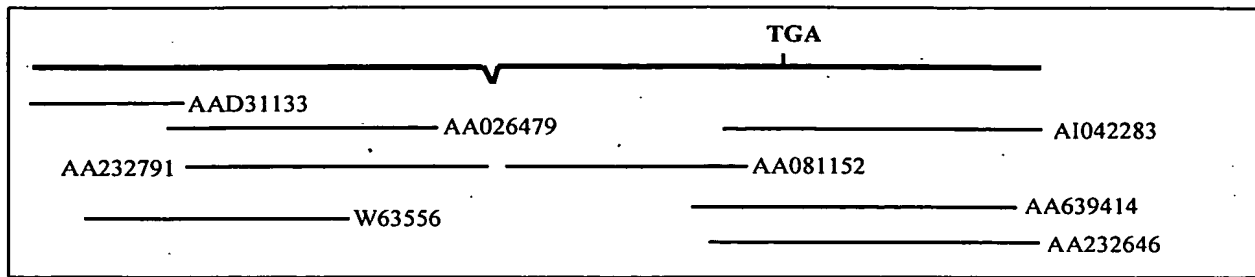


FIGURE 11

# Cloning of HUVEC SK

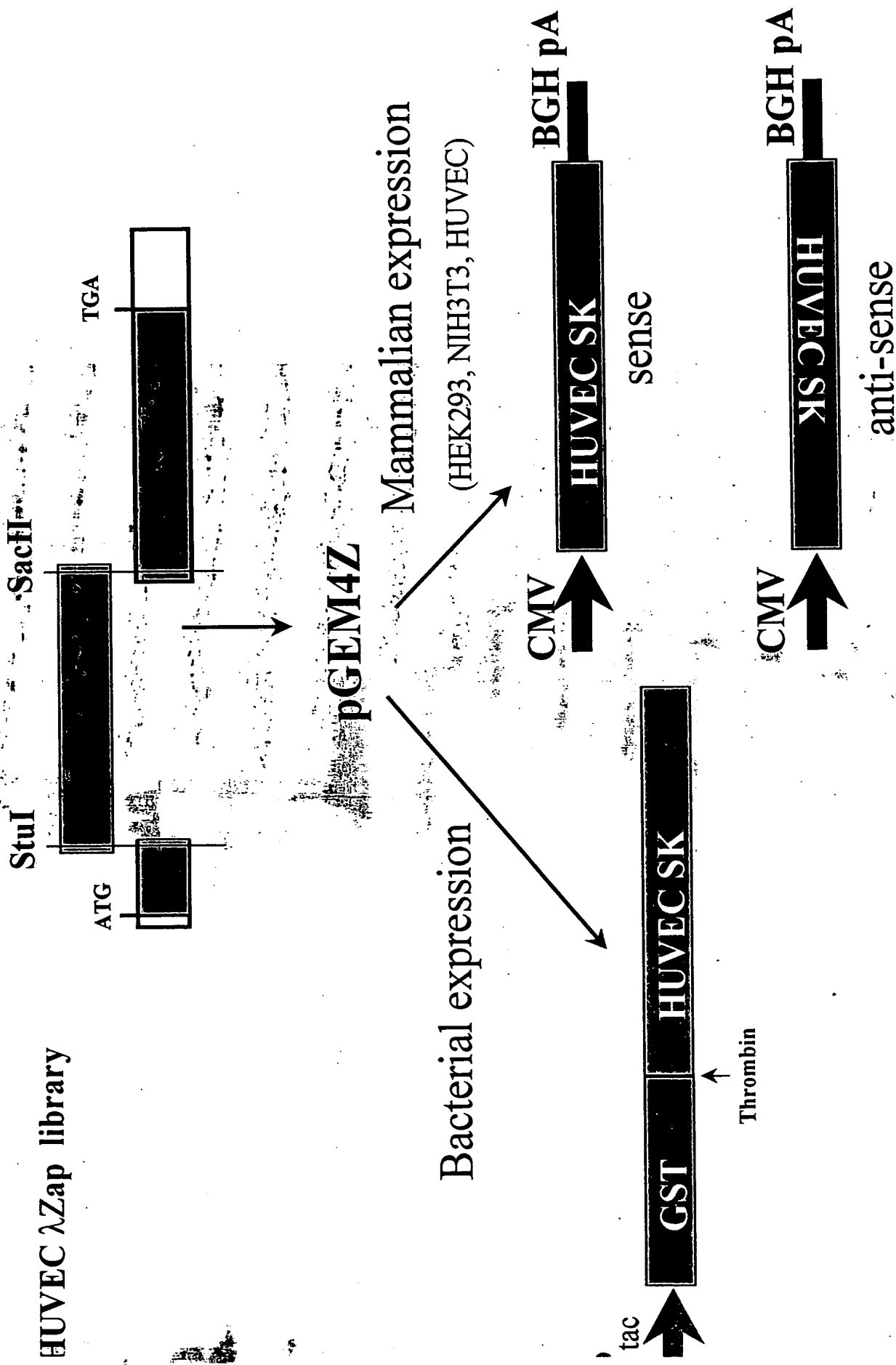


Figure 12.

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1          M D P A G G P R G V 10
1 gaattcggcagcaggagccgcgggtcgaggttATGGATCCAGCGGGCGGCCCCGGGGCG 60

11  L P R P C R V L V L L N P R G G K G K A 30
61 TGCTCCCGCGGGCCCTGCCGCGTGTGGTGTCTGCTGAACCCGCGCGGCGCAAGGGCAAGG 120

31  L Q L F R S H V Q P L L A E A E I S F T 50
121 CCTTGCAGCTCTTCCGGAGTCACGTGCAGCCCCCTTTGGCTGAGGCTGAAATCTCCTTCA 180

51  L M L T E R R N H A R E L V R S E E L G 70
181 CGCTGATGCTCACTGAGCGGCGGAACACGCGCGGGAGCTGGTGCGGTTCGGAGGAGCTGG 240

71  R W D A L V V M S G D G L M H E V V N G 90
241 GCCGCTGGGACGCTCTGGTGGTCATGTCTGGAGACGGGCTGATGCACGAGGTGGTGAACG 300

91  L M E R P D W E T A I Q K P L C S L P A 110
301 GGCTCATGGAGCGGCTGACTGGGAGACGCCATCCAGAAGCCCCTGTGTAGCCTCCCAG 360

111 G S G N A L A A S L N H Y A G Y E Q V T 130
361 CAGGCTCTGGCAACGCGCTGGCAGCTTCCTTGAACCATTATGCTGGCTATGAGCAGGTCA 420

131 N E D L L T N C T L L L C R R L L S P M 150
421 CCAATGAAGACCTCCTGACCAACTGCACGCTATTGCTGTGCCCGGGCTGCTGTACCCCA 480

151 N L L S L H T A S G L R L F S V L S L A 170
481 TGAACCTGCTGTCTCTGCACACGGCTTCGGGGCTGCGCCTCTTCTGTGCTCAGCCTGG 540

171 W G F I A D V D L E S E K Y R R L G E M 190
541 CCTGGGGCTTCATTGCTGATGTGGACCTAGAGAGTGAAGAATATCGGCGTCTGGGGGAGA 600

191 R F T L G T F L R L A A L R T Y R G R L 210
601 TGCGCTTCACTCTGGGCACTTTCTGCGTCTGGCAGCCTTGGCACTTACCGGGGCCGAC 660

201 A Y L P V G R V G S K T P A S P V V V Q 230
661 TGGCTTACCTCCCTGTAGGAAGAGTGGGTCCAAGACACCTGCCTCCCCCGTTGTGGTCC 720

231 Q G P V D A H I V P L E E P V P S H W T 250
721 AGCAGGGCCCGGTAGATGCACACCTTGTGCCACTGGAGGAGCCAGTGCCTCTCACTGGA 780

251 V V P D E D F V L V L A L L H S H L G S 270
781 CAGTGGTGGCCGACGAGGACTTTGTGCTAGTCTGGCACTGTGCACTCGCAGCTGGGCA 840

271 E M F A A P M G R C A A G V M H L F Y V 290
841 GTGAGATGTTTGTGCAACCATGGGCCGCTGTGCAGCTGGCGTCATGCATCTGTTCTAGG 900

291 R A G V S R A M L L R L L A M E K G R 310
901 TCGGGCGGGAGTGTCTCGTGCCATGCTGCTGCGCCTCTCGTGGCCATGGAGAAGGGCA 960

311 H M E Y E C P Y L V Y V P V V A F R L E 330
961 GGCATATGGAGTATGAATGCCCTACTTGGTATATGTGCCCGTGGTCGCCTTCCGCTTGG 1020

331 P K D G K G M F A V D G E L M V S E A V 350
1021 AGCCCAAGGATGGGAAAGGTATGTTTGCAGTGGATGGGAATTGATGGTTAGCGAGGCCG 1080

351 Q G Q V H P N Y F W M V S G C V E P P P 370
1081 TGCAGGGCCAGGTGCACCCAACTACTTCTGGATGGTCAGCGGTGCGTGGAGCCCCCGC 1140

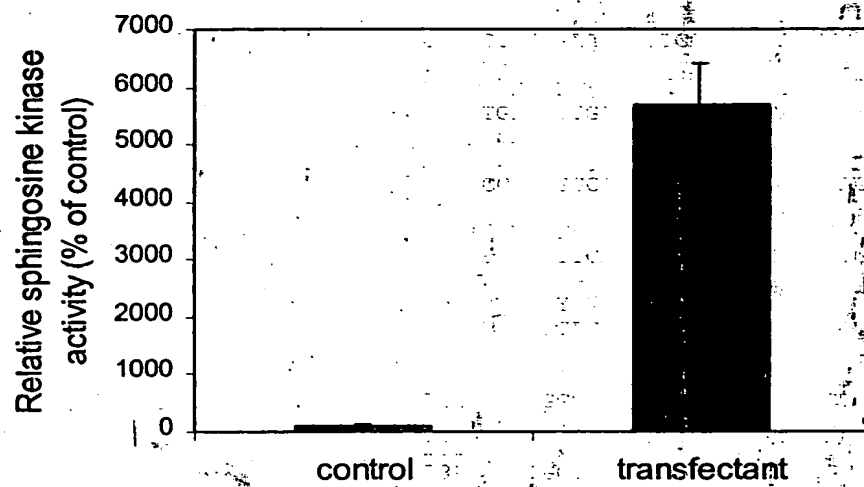
371 S W K P Q Q M P P P E E P L * 384
1141 CCAGCTGGAAGCCCCAGCAGATGCCACCGCCAGAAGAGCCCTTATGAtctagagtcgacc 1200

1201 tgcag 1205

```



Figure 13.



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